Shared Active Sites of Fructose-1,6-bisphosphatase
ARGinine 243 MEDIATES SUBSTRATE BINDING AND FRUCTOSE 2,6-BISPHOSPHATE INHIBITION*

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The active site of pig kidney fructose-1,6-bisphosphatase (EC 3.1.3.11) is shared between subunits, Arg-243 of one chain interacting with fructose-1,6-bisphosphate or fructose-2,6-bisphosphate in the active site of an adjacent chain. In this study, Arg-243 was replaced by alanine using techniques of site-specific mutagenesis and the cloned pig kidney enzyme expressed in Escherichia coli. Compared with wild-type enzyme, kinetic parameters of the altered enzyme characterizing catalytic efficiency, magnesium binding, and inhibition by AMP differed but by less than an order of magnitude; affinity for substrate fructose 1,6-bisphosphate was 10-fold poorer, and affinity for inhibitor fructose 2,6-bisphosphate was 1000-fold poorer. Molecular dynamics simulations were undertaken to determine possible alterations in active sites of the enzyme due to replacement of Arg-243 by Ala and suggested that in the mutant enzyme loss of one cationic group leads to reorganization of the active site especially involving lysine residues 269 and 274. The differences in properties of the mutant enzyme indicate the key importance of Arg-243 in the function of fructose-1,6-bisphosphatase and confirm on a functional basis the shared active site in this important metabolic enzyme.

Metabolic control of fructose-1,6-bisphosphatase (EC 3.1.3.11) plays a key role in regulation of gluconeogenesis. The physiological regulators 5'-AMP and fructose 2,6-bisphosphate negatively modulate Fru-1,6-Pase1 activity and, in reciprocal fashion, positively affect the activity of phosphofructokinase, a control point in glycolysis (for a recent review, see Ref. 1). Investigations of the mechanism of regulation of Fru-1,6-Pase by these effectors, and of the catalytic mechanism of hydrolysis of fructose 1,6-bisphosphate, have been extended to the structural level by the availability of crystal structures for pig kidney Fru-1,6-Pase (2) and complexes of this enzyme with the substrate fructose 1,6-bisphosphate displays hyperbolic kinetics at neutral pH, with partial inhibition at high substrate concentrations, whereas plots of reaction velocity versus cofactor magnesium concentration are sigmoidal (9). Inhibition by AMP is cooperative, with a Hill coefficient near 2 (10, 11), while Fru-2,6-P2 binds to the active sites of the enzyme (2) in competition with the substrate Fru-1,6-P2 (12). The AMP binding site on each subunit is 28 Å distant from the active site (4), and metal-binding sites are located between the Fru-1,6-P2 and AMP domains (13).

DNA coding for mammalian Fru-1,6-P2ase has been cloned from pig kidney (7), pig liver (14), rat liver (8, 15), and human liver (16). Amino acid residues suggested to have functional importance based on analysis of the crystal structure of pig kidney Fru-1,6-P2ase have been modified using techniques of site-specific mutagenesis and comprise some of those interacting at each of the three ligand binding sites. Lys-274 (17), using the numbering system of pig kidney enzyme, interacting with substrate at the active sites, Glu-97, Glu-280, Asp-118, and Asp-121 (16, 18) at the metal-binding sites, and Glu-29 and Thr-31 (19) at the AMP-binding sites have been studied.

The x-ray structure of pig kidney enzyme shows that Arg-243 from one subunit interacts with the 6-phosphate portion of sugar phosphates (2) in the active site of an adjacent subunit (illustrated in Fig. 1). To assess the importance of this interaction for catalysis or inhibition of Fru-1,6-P2ase, we have used site-specific mutagenesis to replace Arg-243 with alanine in the cloned pig kidney enzyme (7). Mutant enzyme (R243A) was expressed in Escherichia coli, and consequences of this mutation on the kinetic parameters of Fru-1,6-P2ase were examined. Only by study of the pig kidney enzyme can the results of mutagenesis of Fru-1,6-P2ase be interpreted with respect to the available three-dimensional structure.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, all components of the enzyme assay, reagents, and buffers were from Sigma. Restriction enzymes were purchased from New England Biolabs, Inc.

Construction of Wild-type and Mutant Enzyme Expression Systems—Complementary DNA for pig kidney Fru-1,6-P2ase was previously cloned from a 1.3-kilobase BstXI fragment inserted into the vector pCDNA II (7). A HindIII-XhoI fragment was excised from this plasmid and cloned into the vector pUC119 (20). Sequencing of mutations in the coding region of the plasmid was simplified because it contained the M13 intergenic region allowing isolation of single-stranded DNA after coinfection with a helper phage such as M13KO7. A site for restriction enzyme Ndel was introduced at the translation start site by site-specific mutagenesis without altering the amino acid coding sequence.

Site-specific Mutagenesis—Oligonucleotides used for site-specific mutagenesis and sequencing by the dyeoxyside procedure were obtained from Operon Technologies or synthesized using an Applied Biosystems 381A DNA synthesizer and purified by high performance liquid chromatography employing a DuPont Zorbax Oligo ion-exchange column. Site-specific mutagenesis was performed by the Kunkel method (21, 22). Ureac-containing single-stranded DNA was obtained by infection of

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1 The abbreviations used are: Fru-1,6-P2ase, fructose-1,6-bisphosphatase; Fru-1,6-P2, fructose 1,6-bisphosphate; Fru-2,6-P2, fructose 2,6-bisphosphate; R243A, the mutant pig kidney fructose-1,6-bisphosphatase with alanine in place of arginine at position 243.
0.8 liters of culture medium were suspended in 160 ml of chem, leupeptin (2.5 µg/ml), and lysozyme (0.1 mg/ml) (14). The suspension was sonicated to release enzyme, and then MgCl₂ was added to a concentration of 1-2 mg/ml. MgCl₂ was added to a concentration of 10 mM, and the solution was stirred at room temperature (29). Over 20 min, a volume of 50% (v/v) solution of polyethylene glycol (average molecular weight, 3350), in 50 mM potassium phosphate buffer, pH 7.0, was added dropwise to 20% (v/v) Concentration. After cooling the suspension on ice for 10 min, insolubilized enzyme was collected by centrifugation. 

Fru-1,6-P₂ase was redisolved to yield 5 mg of protein/ml using a solvent mixture of 8 volumes of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM disodium EDTA and 1 mM dithiothreitol, plus 2 volumes of glycerol and stored at −70°C. 

Fru-1,6-P₂ase Assays and Data Analysis—A spectrophotometric, coupled enzyme assay was employed to measure Fru-1,6-P₂ase activity (30). Standard conditions (2 mM magnesium chloride, 70 µM Fru-1, 6-P₂) were used to determine specific activity. Digital absorbance values were collected every 6-smin interval and analyzed by V. Villaret and W. N. Lipscomb. Molecular dynamics (Langevin) were calculated at 0.001-ps intervals for 2.5 ps after 0.25 ps. 

Other Methods—Protein concentration was determined by the Lowry method; protein was collected by precipitation with deoxycholate-trichloroacetic acid (35). Bovine serum albumin was used as the standard. SDS-polyacrylamide gel electrophoresis was used to judge enzyme homogeneity (36). Concentrations of Fru-1,6-P₂, and NADP were checked by performance in the coupled assay, AMP using the ADE3 kit from Novagen, Inc. The lysogenized host was similarly purified to a specific activity of 18.9 units/mg. Both potentials were screened for ability to support induction of Fru-1,6-P₂ase gene using the ADE3 kit from Novagen, Inc. The lysogenized host was similarly purified to a specific activity of 18.9 units/mg. 

RESULTS

Enzyme Isolation—Eighteen mg of R243A enzyme was obtained from 0.8 liter of cell culture. Half of the activity of the clarified lysate was recovered in purified enzyme, with a 9-fold increase in specific activity to 9.4 units/mg. Wild-type enzyme was similarly purified to a specific activity of 30 units/mg. Both
preparations appeared homogeneous on SDS-polyacrylamide gel electrophoresis; in particular, there was no band of molecular weight near 29,000, which results from proteolytic fragmentation of single-chain subunits (38). The enzymes were stable during storage for at least several months despite repeated thawing and freezing.

**Enzymatic Activity of the Two Fru-1,6-P$_2$ase—**Kinetic parameters that characterize catalytic activity of the two enzymes are presented in Table I. Both enzymes displayed hyperbolic dependence of activity on substrate concentration. Only data to 70 mM Fru-1,6-P$_2$ were used in determining Michaelis-Menten parameters for wild-type enzyme since activity was partially inhibited above that substrate concentration. For example, at 1 mM Fru-1,6-P$_2$, the rate of product formation was half of that at 70 mM. This extent of inhibition was similar to that seen by other investigators (10). The R243A enzyme did not demonstrate substrate inhibition up to 1 mM Fru-1,6-P$_2$ and higher concentrations were not tested. The R243A enzyme had both a higher $K_{m}$ and a lower $V_{max}$ than did wild-type enzyme. Both enzymes displayed sigmoid dependence of activity on magnesium concentration. The R243A enzyme had less avidity for Mg$^{2+}$ compared with the wild-type Fru-1,6-P$_2$ase (see Fig. 2). Data at several concentrations of substrate and Mg$^{2+}$ cofactor were fit to the model proposed by Chen et al. (19). Parameter estimates from global fits were $K_m = 2.0 \pm 0.3$ mM, $k_{cat} = 19.0 \pm 0.4$ s$^{-1}$, and $K_i = 0.19 \pm 0.02$ mM for the wild-type enzyme and $K_m = 22.2 \pm 2$ mM, $k_{cat} = 19.2 \pm 0.2$ s$^{-1}$, and $K_i = 0.56 \pm 0.08$ mM for the R243A enzyme, where $K_m$ is the square of magnesium concentration yielding half-maximal activity.

Since the R243A enzyme required higher substrate and cofactor concentrations to achieve maximum activity than did the wild-type enzyme, in assays of inhibitors to generate the data in Table I, substrate or cofactor or both were held constant at similar multiples of concentrations yielding half-maximal rates for each enzyme. This permitted comparison of the two enzymes at equal fractions of maximum activity. The R243A enzyme required 6-fold higher concentrations of AMP to be inhibited to the same extent as the wild-type enzyme (Fig. 3). The difference in inhibition parameters of the two enzymes persisted when data were fit to a competitive inhibition model specifically requiring stoichiometries of 2 mol of magnesium and AMP (19), but $K_i$ values were smaller than those determined at high proportions of magnesium. These $K_i$ values were $0.22 \pm 0.03$ and $4.7 \pm 0.5$ mM for wild-type and R243A enzymes, respectively. R243A was 1000-fold less sensitive than wild-type enzyme to inhibition by Fru-2,6-P$_2$ (Table I). The inhibitor was competitive with substrate for each enzyme (Fig. 4).

**Monovalent Cation Activation—**Wild-type enzyme activity was stimulated 1.6-fold upon inclusion of 0.15 mM KCl in assay buffer of neutral pH, whereas activity of the R243A enzyme was reduced to 60% of its KCl-free value (Fig. 5). Monovalent cation also activated wild-type enzyme at alkaline pH, but depression of R243A activity was not evident at the higher pH (Fig. 5). The ratio of activity at pH 7.5 to that at pH 9.3 is typically reported for assays in the presence of KCl; for the wild-type enzyme it was 3.5, for the R243A enzyme, 7.4.

**Molecular Dynamics—**In order to determine possible alterations in the active site of Fru-1,6-P$_2$ase due to the replacement of Arg-243 by Ala, molecular dynamics calculations were utilized. As illustrated in Fig. 6, energy minimization and molecular dynamics simulations suggested that replacement of arginine by alanine at position 243 resulted in substantial alteration in the position of inhibitor Fru-2,6-P$_2$ in the active site of Fru-1,6-P$_2$ase. Orientation of substrate Fru-1,6-P$_2$ was not so markedly altered. Positions of the side chains of Lys-269 and Lys-274 were shifted in the mutant enzyme. In the wild-type structure with Fru-2,6-P$_2$, Lys-274 interacts with both sugar phosphates (2). Our dynamics runs indicated that the interaction of the 2'-phosphate dominates. In addition, Arg-243 formed a salt link only with the 6'-phosphate, and Lys-269 did not interact with Fru-2,6-P$_2$. However, in R243A molecular

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**Table I**

**Kinetic parameters for wild-type and R243A Fru-1,6-P$_2$ases**

Studies of the wild-type enzyme used 14 μM Fru-1,6-P$_2$ and 3 mM magnesium concentrations when the components were held constant, except as noted. Studies of the R243A enzyme used 175 μM Fru-1,6-P$_2$ and 5 mM magnesium concentrations when the components were held constant, except as noted.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (Fru-1,6-P$_2$) (μM)</th>
<th>$V_{max}$ (μM)</th>
<th>Hill coefficient (Mg$^{2+}$)</th>
<th>IC$_{50}$ (AMP) (μM)</th>
<th>$K_i$ (Fru-2,6-P$_2$) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>18.4 ± 0.4$^a$</td>
<td>1.7 ± 0.3$^b$</td>
<td>0.39 ± 0.04</td>
<td>2.1 ± 0.1</td>
<td>3.2 ± 0.3</td>
<td>0.068 ± 0.007</td>
</tr>
<tr>
<td>R243A</td>
<td>8.5 ± 0.2$^a$</td>
<td>35 ± 1$^b$</td>
<td>0.96 ± 0.03$^b$</td>
<td>1.8 ± 0.1$^b$</td>
<td>19 ± 3</td>
<td>101 ± 7</td>
</tr>
</tbody>
</table>

$^a$ 2 mM magnesium.

$^b$ 150 μM Fru-1,6-P$_2$.

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**FIG. 2.** Sigmoid dependence of Fru-1,6-P$_2$ase activity on magnesium concentration. Plots of the R243A enzyme (●) at 150 μM Fru-1,6-P$_2$ and the wild-type enzyme (○) at 14 μM substrate.

**FIG. 3.** Inhibition by AMP, expressed as percentage of inhibitor-free control, of the R243A enzyme (●) at 175 μM Fru-1,6-P$_2$, 5 mM magnesium concentration, and wild-type Fru-1,6-P$_2$ase (○) at 14 μM substrate, 3 mM magnesium concentration.
Arg-243 forms part of the active sites of Fru-1,6-P\(_2\)ase; its distinction is that this residue bridges subunits (see Fig. 1), each of the four active sites being constituted with an Arg-243 from another polypeptide chain (2). All other significant components of each active site, and of the AMP-binding region as well, derive from a continuous polypeptide chain. X-ray crystallographic studies of pig kidney Fru-1,6-P\(_2\)ase indicate that Arg-243 interacts principally with the 6-phosphate group of the inhibitor Fru-2,6-P\(_2\). Such studies had led to the prediction that replacement of Arg-243 by Ala would destabilize 6-phosphate binding and reduce affinity for substrate (3). Functional data obtained via site-specific mutagenesis were used to investigate the prediction.

**Active Sites in Fru-1,6-P\(_2\)ase Are Shared Between Subunits**—Removal of the side chain of Arg-243, donated into an active site from an adjacent chain, had a substantial influence on the function of Fru-1,6-P\(_2\)ase. The R243A Fru-1,6-P\(_2\)ase was a less efficient catalyst and interacted less avidly with substrate Fru-1,6-P\(_2\), with cofactor magnesium and with inhibitors AMP and Fru-2,6-P\(_2\). Reductions in \(K_m\), magnesium affinity and inhibition by AMP were modest; avidity for substrate was more markedly reduced; and, most significantly, inhibition by Fru-2,6-P\(_2\) was greatly decreased. Rate equations proposed to describe catalysis and AMP inhibition (19), admittedly stochastic, appeared to fit our data, and parameters obtained from such fits are not linked to specific substrate or cofactor concentrations as are the values in Table I. However, the kinetic implication of two magnesium ions in the catalytic event has not yet been supported by structural studies, which find only one such cation/active site in the presence of nonhydrolyzed substrate analogues (39).

**Arg-243 Is Important for Fru-2,6-P\(_2\) Binding**—Replacement of Arg-243 by Ala in pig kidney Fru-1,6-P\(_2\)ase caused a 1000-fold weaker interaction of the enzyme with Fru-2,6-P\(_2\) compared with the wild-type enzyme. Lys-274 also interacts with the fructose bisphosphates. Lys-274 interacts more weakly with substrate and inhibitor (2). Removal of this residue in rat liver Fru-1,6-P\(_2\)ase by site-specific mutagenesis also reduces avidity for Fru-2,6-P\(_2\) of 1000-fold (17). Although an equivalent mutation in the pig kidney enzyme has not been studied, the sequence homology between rat liver and pig kidney enzymes suggests that this lysine residue serves a similar function in both. El-Maghrabi et al. (17) concluded that Lys-274 allows Fru-1,6-P\(_2\)ase to distinguish between its substrate and the substrate analog/inhibitor, Fru-2,6-P\(_2\). Whereas the mutation at Lys-274 has a 20-fold higher \(K_m\) for substrate (17), the increase in \(K_m\) was 10-fold for the R243A enzyme. Removal of either Lys-274 or Arg-243 thus appears to lead to similar loss of affinity for both substrate and inhibitor fructose bisphosphates.

**Proper Binding of Fru-1,6-P\(_2\) and Fru-2,6-P\(_2\) Is Dependent on Interactions with Enzyme Active Site Cations**—Interactions of enzyme with fructose bisphosphates, either substrate or inhibitor, involves balancing of charged functionalities between ligand anionic and enzyme cationic groups. In the R243A enzyme, absence of the one cationic group appears to lead to some reorientation of ligand in the active site cavity, with movement of ligand toward the remaining cationic residues, Lys-269 and Lys-274. The structural basis by which replacement of Arg-243, which interacts almost exclusively with the 6-phosphate of both Fru-1,6-P\(_2\) and Fru-2,6-P\(_2\), preferentially reduced the affinity of enzyme for inhibitor Fru-2,6-P\(_2\) may involve greater reorientation of the inhibitor in the active site, compared with the smaller reorientation observed in dynamics simulations of the substrate complex (Fig. 6). Specificity of Lys-274 for the 2'-phosphate of the inhibitor Fru-2,6-P\(_2\) does not appear equal heights for activities at neutral pH in the absence of KCl. One unit represents generation of 1 pmol phosphate/min at ambient temperature (these assays only). Substrate, 70 \(\mu\)M, and magnesium concentration, 5 mM, throughout.

**DISCUSSION**

**FIG. 4.** Dixon plots of inhibition by Fru-2,6-P\(_2\) of the R243A (A) and the wild-type (B) Fru-1,6-P\(_2\)ases. For both enzymes, rates were determined using 7 \(\mu\)M substrate (A) and 14 \(\mu\)M substrate (B).

**FIG. 5.** Hydrolytic activities of wild-type Fru-1,6-P\(_2\)ase (left) at pH 7.5 and pH 9.3 in the presence (filled bars) or absence (open bars) of 150 mM KCl in assay solutions and similar assays with R243A (right). Ordinate values for the two enzymes are scaled to present equal heights for activities at neutral pH in the absence of KCl.

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not seem to be maintained in the R243A enzyme. Molecular dynamics simulations also suggest a functional role for Lys-269 in anchoring of both Fru-1,6-P₂ and Fru-2,6-P₂, interactions that are not observed in the crystal structures. Site-specific mutagenesis experiments at Lys-269 will be necessary to explore the role of this residue in the function of pig kidney Fru-1,6-P₂ase.

Arg-243 May Be Involved in Monovalent Cation Activation—Of 13 arginine residues per Fru-1,6-P₂ase subunit, 3 can be reacted with 2,3-butanedione (40). Derivatization of one of the three leads to loss of activation produced by potassium or ammonium salts (41). Presence of substrate during derivatization prevents the loss of responsiveness. In the present experiments, the R243A enzyme was not activated by monovalent cation as was the wild-type enzyme. In this respect, the mutant enzyme resembled the derivatized enzyme (41); however, R243A differed from derivatized enzyme in some important characteristics. In particular, activity of derivatized enzyme in the absence of added potassium is not different from that of native enzyme (41), whereas R243A had a lower specific activity than wild-type enzyme in assay buffer in the absence of added potassium is not different from that of native enzyme (41), whereas R243A had a lower specific activity than wild-type enzyme in assay buffer.

FIG. 6. Superimposed stereoview of an active site of wild-type (thin lines) and R243A (thick lines) Fru-1,6-P₂ases with Fru-2,6-P₂ (F26P) bound. The coordinates are averages over the last 2 ps of a 2.5-ps dynamics run. All side chains directly interacting with inhibitor in the wild-type x-ray structure are shown. For clarity, water molecules used in the dynamics runs are not shown. The 243 position (either Arg or Ala) is from a different subunit than all other residues. This figure was drawn with the program SETOR (42).

Acknowledgments—We thank V. Villeret and W. N. Lipscomb for providing x-ray coordinates of Fru-1,6-P₂ase with Fru-2,6-P₂ bound.

REFERENCES


Conclusions—Removal of the guanidinium side chain at position 243 of pig kidney fructose-1,6-bisphosphatase results in a functional but impaired enzyme. This mutation at the active site altered avidity for magnesium ion and for inhibitor AMP, the binding regions for which are distant from the substrate binding site. Substrate affinity, and particularly, affinity for competitive inhibitor Fru-2,6-P₂ were more significantly decreased. Modeling of interactions at the active site suggested altered orientation of docking of inhibitor and substrate in the mutant enzyme.
Arg-243 Effects on Fru-1,6-P₂ase Active Site

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